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(54) Title: ORAL VACCINES

(57) Abstract

A complex of an immunogen with a carrier molecule and a method for presentation of the immunogen to mucosal epithelia of a host vertebrate in order to elicit a systemic, cellular, and/or mucosal immune response in the host vertebrate to the complex. The invention also provides processes for the production of the complex. Further the invention provides medicaments containing the complex as well as medicaments which additionally contain dietary molecules. The dietary molecules provides a means of selectively modulating the magnitude and/or type of immune response to the complex of the medicament. The invention provides means for inhibiting gonadal function in a mammal as well as for selectively modulating cellular immune response to a complex according to the invention.

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"ORAL VACCINES"

TECHNICAL FIELD

The present invention relates to the specific stimulation of serum and secretory antibodies through mucosal presentation of antigens.

BACKGROUND ART

A number of infections in mammals have sufficient deleterious effects on the mammal to warrant vaccination against the particular antigen responsible for the infection. Therefore, vaccination programmes are instituted whereby the mammal is antigenically challenged with an antigen so that an immune response is elicited to confer immunity to the mammal.

Administration of the antigen to the mammal may be through a number of routes including injection intramuscularly (i.m.), subcutaneously (s.c.), or through oral administration (per os). I.m. or s.c. injection of antigen suffers from the disadvantages that relatively specialized skills are required, it is difficult to undertake on a large scale, it is expensive, and a number of side reactions can occur to either the immunizing antigen or to the emulsifying reagent in which it is presented. Oral administration of vaccines is by contrast relatively problem free except insofar as oral feeding of a number of antigens requires relatively large quantities of antigen as the amount of material that is actually absorbed and capable of stimulating an effective immune response is usually low. Thus the amount of antigen required for oral immunization generally far exceeds that required for systemic induction of immunity. There is also one major disadvantage to the oral presentation of the large quantities of antigen required to produce an antibody response and that is that feeding of these large quantities of antigen often leads to the induction of systemic tolerance (Tomasi, 1980; Mowat, 1985; Mowat and Parrot, 1983; Ngan & Kind, 1978; Hanson et al., 1979; Richman et al., 1978; Rothberg et al., 1973).

Evidence to date suggests that in general the mechanism by which antigen is taken up by the small intestine, following oral feeding, is primarily via non-specific sampling of the contents of the gut lumen by "M" cells which overlie the Peyer's Patches and other lymphoid clusters of the GALT (gut-associated-lymphoid tissue) (Bland and Britton, 1984). The subsequent sensitization of local lymphocyte populations

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leads to the generation of local IgA immune responses plus the sensitization of IgG suppressor cells with concomitant suppression of serum IgG responses (Tomasi, 1980; Mowat, 1985; Mowat and Parrot, 1983; Ngan & Kind, 1978; Hanson et al, 1979; Richman et al, 1978; Rothberg et al, 1973).

It is therefore apparent that the site of antigen uptake, whether it be through the Peyer's Patches or the villous epithelium, and quite probably the amount of antigen administered, dictates the type of immune response generated by orally administered antigen. The question arises then as to whether there are any other antigens apart from cholera toxin which exhibit the ability to specifically prime the mucosal immune system upon oral challenge and/or to stimulate the humoral immune response in a dose dependant manner without inducing systemic tolerance and without the need for excessive doses of antigen.

With this view in mind we decided to investigate the possible potential of certain adhesive molecules, which have been implicated in the initial attachment of a number of intestinal pathogens, to stimulate the immune response when orally administered. These surface antigens which confer adhesive properties to a number of strains of enterotoxigenic *E. coli* (ETEC) have been identified as nonflagellar, filamentous proteinaceous appendages, or pili (Gaastra & de Graaf, 1982). Examples include the CFA I and CFA II antigens of human ETEC strains and the K88, K99, F41 and 987P pili of animal ETEC strains (Gibbons et al, 1975; Evans & Evans, 1978; Levine et al., 1980; Morgan et al., 1978; de Graaf & Roorda, 1982). In addition, we have examined the ability of a number of other proteins which have no apparent role in colonization to prime the immune system upon oral feeding. These antigens included a number of lectins a serotypic antigen of *S. typhimurium* (the type "i" flagella), inactivated flu virus and *S. typhimurium* endotoxin (LPS). Oral priming was compared to the response generated to wholly intramuscular challenge (i.m.).

Thus, the aim of these studies was to provide a method whereby the uptake of an immunogen or antigen by the gastrointestinal tract mucosa is improved to the extent that it is possible to elicit serum and secretory antibodies by oral feeding of low doses of the immunogen without the induction of oral tolerance.

Accordingly the invention describes a group of molecules

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(mucosal immunogens) which when fed lead to the production of serum antibodies to these proteins at comparable levels to those obtained by intramuscular injection of these molecules. Furthermore when larger quantities of these antigens are fed there is a concomitant ⁵ increase in the stimulation of the production of mucosal antibodies to the immunizing molecules.

In a further aspect of this invention a process is described whereby the antibody response generated to the orally fed molecules can be augmented or changed by the co-feeding of a number of dietary ¹⁰ molecules.

In another aspect of this invention a process is described whereby a hapten or protein can be coupled to a mucosal immunogen and the complex of which, when fed, results in the production of ¹⁵ antibodies to the hapten or coupled protein.

ABBREVIATIONS

| | | | |
|-----|----------|---|-----------------------------------------------------|
| 1. | Ab | - | Antibody |
| 2. | BSA | - | bovine serum albumin |
| 3. | ConA | - | Concanavalin A |
| 4. | DNP | - | dinitrophenyl |
| 5. | ELISA | - | Enzyme linked immunosorbent assay |
| 6. | ETEC | - | enterotoxigenic <i>E. coli</i> |
| 7. | GALT | - | gut associated lymphoid tissue |
| 8. | HA | - | hydroxy apatite |
| 9. | IM | - | intra muscular |
| 10. | LRH | - | luteinizing hormone releasing hormone |
| 11. | LPS | - | lipopolysaccharide |
| 12. | LT-B | - | heat labile toxin of enterotoxigenic <i>E. coli</i> |
| 13. | O/N | - | overnight |
| 14. | per os | - | oral administration |
| 15. | PS | - | polysaccharide |
| 16. | RT | - | room temperature |
| 17. | SC | - | subcutaneous |
| 18. | SDS-PAGE | - | SDS - polyacrylamide gel electrophoresis |
| 19. | TCA | - | trichloracetic acid |

DISCLOSURE OF INVENTION

In a first form the invention provides a complex comprising ³⁰ an immunogen; linked to a carrier molecule which is capable of

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specifically interacting with the mucosal epithelium of a vertebrate host, wherein both the immunological activity of the immunogen and the capacity of the carrier molecule to specifically interact with the mucosal epithelium of the vertebrate host is substantially maintained, and said complex is capable of eliciting a systemic, cellular and/or mucosal immune response in the vertebrate host.

Preferred immunogens according to the invention include: all, part, analogues, homologues, derivatives or combinations thereof and a hormone, therapeutic agent, antigen or hapten. These immunogens include hormones such as LHRH (luteinising hormone releasing hormone), FSH, HGH and Inhibin; allergens such as grass pollens (for instance barley and couch), weed pollens (eg. clover, dock), tree pollens (eg. ash, cypress), plant pollens (eg. broom), epithelia (eg. cat hair, dog hair, pig hair) and house dust, wheat chaff and Kapok; immunogens for vaccines against agents such as influenza, measles, Rubella, smallpox, yellow fever, diphtheria, tetanus, cholera, plague, typhus, BCG, haemophilus influenzae, Neisseria catarrhalis, Kelbsiella pneumonia, pneumococci and streptococci especially S. mutans; and pili including pili derived from E. coli, N. gonorrhoeae, N. meningitis, N. catarrhalis, Yersinia spp, Pseudomonas aeruginosa, Pseudomonas spp, Moraxella bovis, Bacteroides nodosus, Staphylococci spp, Streptococci spp and Bordetella spp.

Preferred carrier molecules include bacterial adhesins such as 987P, K99, CFAI, CFAII, K88 or F41; viral haemagglutinins such as from influenza, measles, Rubella, smallpox or yellow fever viruses; toxins or binding subunits thereof such as LTB, ricin, abrin, diphtheria toxin, modecin, tetanus toxin and others of similar structures; and lectins whether from plant or other origin. Lectins include for example conconavalin A, Pokeweed mitogen or lectins from Lens culinaris, Helix pomatia, Glycine max, Arachis hypogea, or Ulex europeus or Abrin, Asparagus pea, Broad bean, Camel's foot tree, Castor bean, Fava bean, Green marine algae, Hairy vetch, Horse gram, Horse shoe crab, Jack bean, Japanese wisteria, Jequirity, Scotch laburnum, Lima bean, Limulin, Lotus, European mistletoe, Mung bean, Osage orange, Pagoda tree, Garden pea, Potato, Red kidney bean, Red marine algae, Siberian pea tree, edible snail, garden snail, Spindle

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tree, Sweet pea, Tomato, wheat germ or winged pea.

In a preferred embodiment of the invention there is provided a complex which comprises luteinising hormone releasing hormone and LTB.

In another form the present invention provides a process for the production of a complex as described above which process comprises:

- (a) reacting the immunogen with the carrier molecule to form said complex; and
- (b) chemically modifying the immunogen to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex; or
- (c) chemically modifying the carrier molecule to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex; or
- (d) chemically modifying the immunogen and the carrier molecule to provide functional groups capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;
- (e) reacting the immunogen with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex;
- (f) reacting the carrier molecule with at least one linking agent and reacting the immunogen and the carrier molecule to form said complex;
- (g) reacting the immunogen and the carrier molecule with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex; or
- (h) a combination of any of the preceding process steps.

In another form the invention provides a process which comprises: providing a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA; transforming a host with said recombinant DNA molecule so that said host is capable of expressing a hybrid proteinaceous product which comprises said complex; culturing said host to obtain said expression; and collecting said hybrid proteinaceous product.

Alternatively the invention provides a process for the production of a complex which process comprises:

- (a) chemically synthesising the immunogen and/or the carrier

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molecule, and forming the complex by chemical reaction, or (b) synthesising a hybrid peptide comprising amino acid sequences of the immunogen and the carrier molecule. Preferably the peptide is prepared by solid phase, enzymatic or manual peptide synthesis.

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In a preferred form of the invention the synthesised immunogen or carrier molecule whilst bound to the resin of the solid phase peptide synthesiser is coupled to the carrier molecule or immunogen respectively.

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In another form of the present invention there is provided a transformant host transformed with a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequences of all, part, an analogue, homologue, derivative or combination thereof, of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of all, part, an analogue, homologue, derivative or combination thereof of the carrier molecule, and vector DNA.

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In a preferred form of the invention the transformant host is a Gram negative or Gram positive bacterium, a yeast, fungus or a higher eukaryotic cell. In one preferred form of the invention, said host is the E.coli. A culture of a transformant microorganism falling within the scope of the present invention had been deposited with the American type culture collection and has been designated the number

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In a further form the invention provides a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA. In a preferred embodiment of this form of the invention, the vector DNA is plasmid DNA but alternative vectors are envisaged within the scope of the present invention and these include viruses, bacteriophages and cosmids. In one preferred form of the invention there is provided plasmid pBTAK66 which when a host cell is transformed with said plasmid will yield a proteinaceous product which includes a polypeptide falling within the scope of the present invention.

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In a further form of the invention there is provided a polynucleotide sequence which comprises a first hybrid polynucleotide

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sequence which acts as a coding sequence for a fusion product comprising an amino acid sequence of an immunogen fused to an amino acid sequence of a carrier molecule, a polynucleotide sequence sufficiently related to said first hybrid polynucleotide sequence so as to hybridize to said first hybrid polynucleotide sequence, and a polynucleotide sequence related by mutation, including single or multiple base substitutions, deletions, insertions and inversions, to said first hybrid polynucleotide sequence or hybridizing sequence or a polynucleotide sequence which on expression codes for a polypeptide which displays similar biological or immunological activity to said fusion product.

Preferably the polynucleotide sequence is one wherein the first hybrid polynucleotide sequence acts as a coding sequence for the amino acid sequence of all, part, an analogue, homologue, derivative or combination thereof of LHRH fused to the amino acid sequence of a carrier molecule, more preferably LTB.

In a further form of the invention there is provided a medicament which comprises a complex according to the invention together with a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers and diluents include typical carriers and diluents such as tablets, aqueous solutions, sodium bicarbonate solutions and similar diluents which neutralise stomach acid or have similar buffering capacity, glycols, oils, emulsions, oil-in-water or water-in-oil emulsions, and include medicaments in the form of emulsions, gels, pastes and viscous colloidal dispersions. The medicament may be presented in capsule, tablet, slow release or elixir form or as a gel or paste or may be presented as a nasal spray and in this form may be in the presence of an aerosol. Furthermore, the medicament may be provided as a livestock feed or as foodstuffs suitable for human consumption.

The present inventors have also found that co-administration of certain dietary molecules with a complex of the present invention can selectively modulate the magnitude and/or type of the immune response to the immunogen of the complex.

Accordingly the present invention further provides a medicament which comprises the complex of the present invention together with a dietary molecule which dietary molecule can selectively modulate the

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magnitude and/or type of the immune response to the immunogen of the complex.

The dietary molecule envisaged by the present invention include, basic, neutral and acidic amino acids, such as arginine, histidine, lysine, alanine, cysteine, cystine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, aspartic acid, glutamic acid; water soluble and

insoluble vitamins, such as thiamine, riboflavin, pyridoxal, cyanocobalamin (V_B₁₂), ascorbic acid (V_C), Vit D₂, etc.

Ergosterol, Vit.E, Vit.A, Vit.K etc; sugars including monosaccharides e.g. galactose, mannose, mannitol, sorbitol, glucose, xylose, Allose, altrose, arabinose, digitoxose, erythrose, fructose, lyxose, muramic acid, mannose, pyruvic acid, ribose, tagatose, talose and the amidated and N acetylated, derivatives thereof; oligosaccharides e.g. lactose, maltose, melibiose, sucrose, cellubiose, N,N-diacetyl chitobiose, gentobiose, isomaltose, lactobionic acid, trehalose, turanose; and dietary minerals and co-factors such as manganese, magnesium, zinc, calcium and iron.

The invention also provides a method of presenting a complex of the present invention which method comprises the mucosal administration of a complex of the present invention together with a dietary molecule capable of modulating the magnitude and/or type of immune response of the immunogen.

The invention also provides the oral administration of the medicaments of the invention, in order to elicit a response to the active molecule in the host. Such a response in the case where the active molecule is an antigen or hapten may be a systemic and/or a mucosal immune response. In the case where the active molecule is LHRH or a derivative, analogue, homologue, part or combination thereof, of LHRH, the response will be inhibition of gonadal function in the host. Where the oral medicament incorporates a dietary molecule according to the invention, the invention provides a method for enhancing the host's response to the active molecule which comprises administering such an oral medicament to the host.

35 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the N-terminal amino acid sequence of the 987P pilin subunit, in comparison with the N-terminal amino acid sequence

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of other pilin proteins.

MODES FOR CARRYING OUT THE INVENTIONMaterials

Lectins were purchased from Sigma Chemical Co. Inactivated flu vaccine was purchased from the Commonwealth Serum Labs. (Australia).

Sugars and vitamins were obtained from the following sources:-

Lactose (AR grade) - Ajax Chemicals, Sydney, Australia; Fructose D(-), Mannose D(+), Sorbitol and Xylose D(+) (all AR grade) - B.D.H.

Chemicals Ltd. Poole, England; Melibiose D(+) - Sigma Chemical Co., St. Louis, Miss.; Retinal (Vit A. aldehyde) - Fluka AG, Chemicals, Fabrik

Buchs, Switzerland; Thiamine - HCl (Vit B1), Riboflavin (Vit B2), Pyridoxal (Vit B6), Cyanocobalamin (Vit B12), L-ascorbic acid (Vit C), Ergosterol (Pro Vit D) and dl-a-tocopherol (Vit E) - Sigma Chemical

Co., St. Louis, Miss.

Bacterial Strains and Media

The *E. coli* K99, 987P and LTB strains used in these experiments are listed in Table 1, and were the generous gifts of Dr. Susan Clark, (Molecular Biology Laboratory Biotechnology Australia). Cultures were grown at 37C (unless otherwise specified) with shaking in Luria broth (LB) with or without 1mM isopropylthio-*n*-D-galactopyranoside (IPTG) as indicated (Table 1). *Salmonella typhimurium* was grown at 37C with shaking in LB plus 0.2% glycerol.

TABLE 1

| Stain | Genetic Marker | Strain | Origin | Phenotype |
|---------|----------------|---------|--------|-----------|
| BTA 595 | p BTA 193 | RB 791 | LTB + | |
| BTA 604 | p BTA 201 | MC 1061 | 987P | |
| BTA 262 | p BTA 106 | ED 8654 | K99 | |

+ Included ~ 1mM IPTG

PURIFICATION OF ANTIGENSPili preparation

E. coli expressing the cloned pili, as detected using radiolabelled antiserum were harvested during logarithmic phase of

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growth. Cultures were heated at 60C for 30 minutes, after which the organisms were pelleted by centrifugation (3,000 x g, 30 min 4C). The supernatant was examined for pili content by 12.5% SDS-PAGE using a modification of the method of Laemmli (Laemmli, 1970; Salit et al., 1980).

5 K99 purification:- The culture supernatant was adjusted to pH 9.7 with 10N NaOH and stirred at room temperature (R.T.) for 10 minutes. The resultant precipitate containing pili was recovered by centrifugation (3,000 x g, 30 min 4C) and resuspended in 100ml 10 distilled H₂O (dH₂O) pH 7.2. This procedure was repeated twice.

10 987P Purification:- Procedures used were as detailed above except that pili precipitation was achieved by adjusting the pH to 3.9 with glacial acetic acid.

Hydroxyapatite chromatography

15 Hydroxyapatite (HA) (DNA grade Bio-Gel HTP, Bio-Rad) was gently swollen in an excess of dH₂O and after a brief period (< 2 min.) fines were decanted gently. Fresh dH₂O was added and used to gently resuspend the gel after which fines were decanted again. This procedure was repeated several times. A column (30 x 5 cm) was filled 20 with a slurry of approximately 30% HA and allowed to settle by gravity. Tight packing was then achieved by passing dH₂O through the column at a flow rate of 16ml/hr until the gel bed surface was stationary. Samples (100 ml) of either K99 or 987P were applied at flow rates not exceeding 30ml per hour. The column was then washed 25 with dH₂O until no protein was detected in the flow through as detected at 280 nm. Pili were eluted at a flow rate of 30ml/hr using a linear gradient of 15 to 250 mM sodium phosphate pH 7.5. Fractions were collected and examined by SDS-PAGE. The pili peak was recovered and pooled.

Ion-exchange Chromatography

30 Pooled fractions of K99 and 987P pili (from the HA chromatography) were reprecipitated with NaOH (pH 9.7) or glacial acetic acid (pH 3.9) respectively. After centrifugation (3,000 x g, 10 min), the pellets containing pili were resuspended in 50 mM Citrate buffer, pH 5.5 (K99) and 50 mM Tris.HCl pH 8.5 (987P) prior to loading 35 on the ion-exchange columns equilibrated with the same buffers. K99 and 987P were loaded onto CM and DEAE columns respectively at a flow

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rate of 100ml/hr, washed with 2 volumes of loading buffer and the pili eluted using a linear gradient from 10 mM to 0.5 M NaCl in the equilibration buffers. Fractions were examined by SDS-PAGE for protein content and LPS contamination, according to the method of Tsai and Frasch (1982).

LTB Purification

Three litres of LTB supernatant was diluted to 61 with dH₂O. The pH was adjusted to 6.5 with glacial acetic acid and loaded onto a 5 x 30 cm column of fastflow CM-Sephadex equilibrated with 10 mM phosphate buffer pH 6.5 at a flow rate of 1.2 l/hr. The column was then washed with 400 mls of 10 mM phosphate buffer pH 6.5 and bound protein eluted with a linear gradient of 10-500 mM NaCl in 10 mM phosphate pH 6.5. Fractions were collected and analysed by SDS-PAGE, the LTB peak was pooled.

Flagellae Isolation

Late log phase cultures of bacteria were pelleted by centrifugation (3,000 x g for 15 mins. at 4C). The cells were resuspended in saline and heated at 60C for 30 minutes, followed by centrifugation (3000 x g, 10 min 4⁰C). The supernatant was precipitated by adding a solution of 100% TCA (w/v) to give a final concentration of 10% (w/v) and spun for 10 min at 1,500 x g 4C. The pellet was resuspended in a small volume of 1 M Tris pH 8.8 and sonicated until in solution. Ethanol was added to a final concentration of 80% (v/v) and the flagellae spun down at 2,000 x g, 10 min at 4C. The pellet was resuspended in acetone, sonicated into suspension and reprecipitated by centrifugation (5,000 x g). Finally the pellet was brought into solution by boiling in 10% SDS and 50 mM EDTA in 10 mM Tris HCl pH 8.0, prior to Sephadex S-200 chromatography.

Flagellae purification

After boiling for 15 min the flagellae were clarified by centrifugation for 5 min in a Beckman benchtop microfuge to remove non-solubilized material. The supernatant was applied to a 2.5 x 80 cm column of Sephadex - S200 (Pharmacia, Fine Chemicals) equilibrated with 20 mM Tris pH 8.8, 0.1% SDS and 10 mM EDTA and eluted using the same buffer. Fractions were collected and analysed by SDS-PAGE. Finally the flagellae peak was pooled and precipitated with 10% (final

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conc.) TCA followed by centrifugation, ethanol and acetone washes as described previously. The final pellet was resuspended in dH₂O.

Lipoplysaccharide (LPS) Purification

Overnight cultures of S. typhimurium were extracted (30 min at R.T.)

5 with 0.5 M CaCl₂ in 20% ethanol (v/v) containing 100 mM Tris-HCl, 10 mM citrate pH 3.0 and 5% Zwittergent 3.12 (w/v) (Calbiochem.). Bacteria were pelleted by centrifugation (3,000 x g, 10 min at 4°C) and the pellet resuspended in 50 mM EDTA pH 8.0. The suspension was stirred vigorously for 30 min at R.T. After removal of the bacteria by centrifugation ethanol was added to the supernatant to a final concentration of 75%. Protein material was pelleted and the supernatant adjusted to 90% ethanol. The precipitate which formed was pelleted and washed with acetone, reprecipitated and finally resuspended in dH₂O. The preparation was assayed for sugar content using the Anthrone reagent (Herbert et al, 1985) and checked for the presence of contaminating proteins using SDS-PAGE. Commercial E. coli LPS (Sigma Chemical Co.) was used as a standard in both assays. Gels were stained for LPS using a silver stain according to the method of Tsai and Frasch (1982).

Preparation of Polysaccharide (PS)

Lipid A was cleaved from the S. typhimurium LPS preparation by incubating the LPS with 1 M glacial acetic acid and heating at 100°C for 2-5 hrs. Lipid A was then removed by centrifugation at 3,000 x g for 10 mins at 4°C.

Description of Purified Antigens

SDS-PAGE analysis of purified K99 and 987P pili preparations revealed the presence of a single band migrating at 17,500 and 20,000 mol. wt. (respectively) under reducing conditions (Fig. 1). This agrees with the published data of Isaacson and others (Isaacson & Richter, 1981; Morris et al, 1980; de Graaf et al, 1981; Fusco et al, 1978). The ease of precipitation of these proteins at pH 9.7 and 3.9 (for K99 and 987P, respectively) suggests that the pI of these two proteins to be around these ranged (see: Isaacson & Richter, 1981; de Graaf et al, 1981). Silver staining of these preparations showed them to contain little (< 1 µg / 100 µg protein) or no contamination with LPS.

Determination of the 987P Amino Terminal Sequence

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Amino terminal micro-sequencing was performed for us by Biotechnology Research Enterprises S.A. Pty. Ltd. Adelaide, South Australia. A 100 n mole sample of 987P purified as described above was assayed. The amino terminal sequence of 987P is compared with the published sequence of K99 and reveals no homology between these two molecules (Fig. 1) (Gaastra and de Graaf, 1982).

Purified LTB, and S. typhimurium flagellae were also found to be free of contaminating LPS and to travel as monomers of apparent molecular weights of 12,500 and 52,000 respectively when examined by SDS-PAGE under reducing conditions.

Silver stained SDS-PAGE gels of purified LPS revealed no detectable protein contamination. Complex sugar content, as assayed by Anthrone reaction, was found to be 2mg/ml. Lipid A free polysaccharide was also found to contain 2 mg/ml polysaccharide and it's failure to move on SDS-PAGE (as revealed in the silver stained gels) showed it to be free of contaminating lipid.

Dinitrophenylation of Antigens

K99, LTB and lectins were dinitrophenylated according to the method of Little and Eisen (1967). Briefly, carriers (in 0.1 M carb/bicarb buffer pH 9.5) were reacted with a 0.1 M solution of DNFB (in Acetone) overnight at R.T. The proteins were then dialysed extensively against the coupling buffer. Previous studies by us have shown that 987P has no free amino groups exposed for coupling so a diamino spacer was first linked to the free carboxyl moieties of the protein as follows: 10 mg of purified 987P was precipitated at pH 3.9 by the addition of glacial acetic acid. The pili were removed by centrifugation at 3,000 x g, 10 min at 4C.. The pellet was resuspended in dH₂O and the pH raised to 6.5 with 1N NaOH. The pili solution was then reacted with 1-ethyl-3-(3-Dimethylaminopropyl) carbodiimide-HCl (EDAC, Bio Rad Laboratories, Richmond, California), to a final concentration of 0.5 mM in the presence of 20 mM 1,2-diaminoethane (BDH Chemicals Ltd. Poole, England), overnight at room temperature (20-23C). The amino-substituted 987P was dialysed for 24 hrs against two changes of 0.1 M carbonate/bicarbonate buffer pH 9.5 before being used in the subsequent conjugation steps. Lectin binding sites were protected during their reaction with DNFB by the addition of the Lectin specific sugars. Thus, 50 mM solutions of

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D-glucose, D-mannose, D-glucose, N-acetyl-D-galactosamine, D-galactose, N-acetyl-D-galactosamine, D-gal(1-3)-D-galN-AC, and L-fucose, were added to the following lectins:—Concanavlin A, Pokeweed mitogen, *Lens culinaris*, *Helix pomatia*, *Phaseolus vulgaris*, *Glycine max*, *Arachis hypogea* and *Ulex europeus*, respectively.

Antigen administration

Female C57BL/6J mice (18-22 gm) were obtained from the Animal

Resources Centre (Perth, Western Australia). All mice were starved for 3-4 hours prior to oral or intramuscular (i.m.) administration of antigens. Mice were fed antigen at appropriate concentrations in 0.5 ml of 0.1M carb/bicarbonate buffer pH 9.5 using a specially prepared feeding needle. Parallel doses of antigen were injected i.m. in 0.1ml of sterile physiological saline, into the left hind leg. Groups of 5 mice receiving antigen either orally or im were given two identical doses of antigen, on day 0 and day 14. A blood sample was taken (approx. 0.5 ml) from the retro-orbital plexus on day 14 and day 21. Mice were then sacrificed by cervical dislocation and gut washes performed on the small intestine in the following manner. The small intestine was carefully removed and a small quantity of washing buffer (1.0 ml, 30 mM Tris.HCl pH 8.8, 0.9% NaCl, 50 mM EDTA plus 1.0% Tween 20) introduced into the lumen of the gut via a blunt ended feeding needle. After gently kneading the intestine the contents were squeezed out through forefinger and thumb. Gut washes so obtained were immediately centrifuged to remove debris and stored at -20C until assayed. Blood samples were allowed to clot at 4C before removal of the serum and storage at -20.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA for the determination of antibody titres was performed as described previously by Russell-Jones et al., (1984).

EXAMPLE 1

Identification of Molecules Active as Mucosal Immunogens

The possible potential of a number of molecules known to possess the capacity to bind to the intestinal mucosa and to stimulate the production of an immune response after oral administration of these molecules was examined. The response generated by these molecules was compared to the response seen after similar feeding of other molecules having no mucosal binding functions.

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As seen in Table 1.1, three broad classes of proteins were detected in these experiments:- those that elicited a serum and intestinal response, K99, 987P, LTB, flu vaccine and the various lectins (Class I) (these shall henceforth be referred to as mucosal immunogens), those that elicited only a serum response (LPS) (Class II) and those that failed to elicit either a serum or intestinal response at the doses tested (Flagellae, BSA and P.S.) (Class III). Within class I antigens 987P was a significantly superior stimulator of IgA antibody (ab) (48.5 ± 1.8) when compared to LTB (12.2 ± 4.4), or K99 (3.2 ± 4.9). In addition 987P also stimulated gastrointestinal IgG (10.8 ± 1.76) to a greater extent than either K99 (3.0 ± 5.3), or LTB (1.0), and only 987P was capable of stimulating serum IgA (10.8 ± 8.8). All four class I antigens stimulated serum IgG to similar degrees (Table 1.1). The class II antigen, LPS, stimulated a small serum IgG response (12.1 ± 1.0) with no concomitant IgA or agastrointestinal reactivity. Finally BSA, flagellae and the polysaccharide moiety of LPS - class III antigens - failed to induce either serum or intestinal IgG or IgA. Representative samples from all three classes; K99, 987P, LTB, LPS and flagellae were administered intramuscularly and screened for both serum and intestinal response (Table 1.2). Class I and II antigens gave similar serum IgG responses yet failed to produce serum IgA or intestinal IgG/IgA Ab responses. Only the anti-LPS serum IgG response appeared significantly improved by i.m. immunisation. Each of 987P, K99 and LTB were further examined in dose response studies after both oral and i.m. administration. As seen in Tables 1.3 and 1.4, 987P yielded consistently higher titers than either K99 or LTB regardless of the route of administration. Interestingly the class I antigen - LTB - displayed a bell shaped dose-response with a plateau maximum between 10 and 50 μ g. None of the other class I antigens produced this effect, nor did the LTB when administered i.m. Oral administration of all class I antigens (Ag) elicited higher levels of intestinal IgA Ab (S-IgA) over the broad range of doses tested. Comparison between the two routes of administration suggests that although i.m. injection consistently gave higher titers, oral administration of mucosal immunogens resulted in comparable levels of antibody production to that obtained by the i.m. route between 10 and 100 μ g of antigen for class I and II antigens.

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Table 1.1
Immune reactions to orally presented antigens

| 5 | Antigen used for immunisation (20 µg does) | Immune response,* day 21. | | | |
|------|--------------------------------------------------|---------------------------|------------|------------|-------------|
| | | Serum | Intestinal | IgG | IgA |
| K99 | | 968 ± 120 | <4 | 3.0 ± 5.2 | 3.2 ± 4.9 |
| 987P | | 776 ± 64 | 10.8 ± 8.8 | 10.9 ± 1.7 | 48.5 ± 1.88 |
| LTB | | 1351 ± 211 | <4 | <4 | 12.2 ± 4.4 |
| 10 | Flue vac | 179 ± 34 | <4 | <4 | <4 |
| | Flagellae | <4 | <4 | <4 | <4 |
| | LPS | 12.1 ± 1.0 | <4 | <4 | <4 |
| | PS | <4 | <4 | <4 | <4 |
| | BSA | <4 | <4 | <4 | <4 |
| 15 | | | | | |
| | Con A** | 666 ± 84 | <4 | nd | nd |
| | FW-mitogen** | 641 ± 119 | <4 | nd | nd |
| | L. culinaris** | 954 ± 48 | <4 | nd | nd |
| | H. pomatia** | 591 ± 127 | <4 | nd | nd |
| 20 | P. vulgaris** | 1378 ± 110 | 4.8 ± 2.3 | nd | nd |
| | G. max** | 1529 ± 65 | 3.1 ± 6.9 | nd | nd |
| | A. hypogea** | 1276 ± 242 | <4 | nd | nd |
| | U. europeus** | 1583 ± 94 | <4 | nd | nd |

* The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 minutes at 37°C. Each value represents the mean value obtained from 5 mice ± 1 standard deviation.

** Each lectin was substituted with 4 DNP molecules/mole lectin.

The ELISA titre represents the anti-DNP response as measured against DNP-BSA.

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Table 1.2

Immune reactions to antigens presented intramuscularlyAntigen used ~~intramuscularly~~ ~~subcutaneously~~ Immune response, * day 21for immunisation Serum Intestinal

| | | Serum | Intestinal | | |
|----|-----------|------------|------------|-----|-----|
| | | IgG | IgA | IgG | IgA |
| 5 | K99 | 1024 ± 94 | <4 | <4 | <4 |
| | 987P | 1552 ± 112 | <4 | <4 | <4 |
| 10 | LTB | 1782 ± 100 | <4 | <4 | <4 |
| | Flagellae | 1595 ± 227 | <4 | <4 | <4 |
| | LPS | 388 ± 58 | <4 | <4 | <4 |

* See Table 1.1

Table 1.3

Dose response to orally presented antigens

Antigen

Immune response*, day 21, per dose (ug)

0.1 1.0 10 50 100 1,000

Serum IgG

| | | | | | | |
|------|-----|-----|------|------|------|------|
| K99 | 1.0 | 4.0 | 28 | 675 | 1351 | 4096 |
| 987P | 4.0 | 14 | 84 | 588 | 1024 | 3104 |
| LTB | 9.0 | 194 | 1351 | 1331 | 891 | 891 |

Serum IgA

| | | | | | | |
|------|----|----|----|----|-----|------|
| K99 | <4 | <4 | <4 | <4 | <4 | <4 |
| 987P | <4 | <4 | <4 | <4 | 9.6 | 18.3 |
| LTB | <4 | <4 | <4 | <4 | <4 | 32 |

Intestinal IgG

| | | | | | | |
|------|----|----|----|-----|-----|-----|
| K99 | <4 | <4 | <4 | <4 | <4 | <4 |
| 987P | <4 | <4 | <4 | <4 | <4 | <4 |
| LTB | <4 | <4 | <4 | 4.0 | 4.0 | 8.0 |

Intestinal IgA

| | | | | | | |
|------|----|----|-----|-----|------|-----|
| K99 | <4 | <4 | <4 | 4.0 | 16.7 | 87 |
| 987P | <4 | <4 | 9.1 | 54 | 84 | 147 |
| LTB | <4 | <4 | <4 | 4.0 | 4.0 | 8.0 |

* see Table 1.1.

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Table 1.4
Dose response to intramuscularly presented antigens

Antigen **Immune response*, day 21, per dose (ug)**

| | | 0.1 | 1.0 | 10 | 50 | 100 | 1,000 |
|----|----------------|-----|-----|------|------|------|-------|
| 5 | | | | | | | |
| | Serum IgG | | | | | | |
| | K99 | 256 | 445 | 776 | 1351 | 1776 | 5104 |
| | 987P | 256 | 891 | 1024 | 3104 | 4096 | 18820 |
| | LTB | 64 | 588 | 1176 | 2702 | 4096 | 8192 |
| 10 | | | | | | | |
| | Serum IgA | | | | | | |
| | TK99 | <4 | <4 | <4 | <4 | <4 | <4 |
| | 987P | <4 | <4 | <4 | <4 | <4 | <4 |
| | LTB | <4 | <4 | <4 | <4 | <4 | <4 |
| 15 | | | | | | | |
| | Intestinal IgG | | | | | | |
| | K99 | <4 | <4 | <4 | <4 | <4 | <4 |
| | 987P | <4 | <4 | <4 | <4 | <4 | <4 |
| | LTB | <4 | <4 | <4 | <4 | 7.0 | 16 |
| 20 | | | | | | | |
| | Intestinal IgA | | | | | | |
| | K99 | <4 | <4 | <4 | <4 | <4 | 4.6 |
| | 987P | <4 | <4 | <4 | <4 | 4.0 | 6.0 |
| | LTB | <4 | <4 | <4 | <4 | 16.0 | 11.1 |

* See Table 1.1.

EXAMPLE 2

Effect of dietary molecules on the immune response to mucosal immunogens upon oral presentation.

Initial studies in our laboratory using outbred Swiss male mice suggested that it was possible to alter the immune response to orally presented antigens by the co-feeding of certain dietary molecules. Accordingly, the mucosal immunogens, K99, 987P and LTB were fed to mice in the presence of a number dietary sugars and vitamins. It was reasoned that as the different antigens were known to bind to different molecules on the surface of the intestinal epithelium, and as it is known that there is a change in the distribution of glycoproteins and glycolipids throughout the length of the gut as well as a change in distribution of absorptive cells, it might be possible to stimulate the uptake of molecules bound to these cells by presenting the antigens in the presence of the specific dietary molecule normally taken up by these cells. An extension to this

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argument would be that the profile of stimulatory molecules would change from antigen to antigen.

The results represented in tables 2.1, 2.2 and 2.3 demonstrate that although most of the vitamins and sugars have had some effect in modulating the immune response to K99, 987P and LTB, some dietary molecules appear to be selective as to which mucosal immunogen they appeared to influence but also as to whether they induced primarily a secretory or serum response. Thus, the serum antibody response to K99 was significantly increased ($P < 0.05$) by co-administration with Vit B12 or Melibiose, unchanged when given with Vit B2, Vit D, Vit E, Fructose or Mannose and decreased to varying extents with Vit A, Vit B1, Vit B6, Vit C, Lactose, Sorbitol and Xylose (Table 2.1).

In contrast, the serum Ab response to oral 987P (Table 2.2) was elevated when 987P was co-administered with Vit B6, Vit B12, Vit C, Vit E, Fructose or Mannose, it was unchanged with Vit A, Vit B1, Vit B2, Lactose, Melibiose, Sorbitol and Xylose, and decreased in the presence of Vit D. LTB on the otherhand, displayed a unique profile for the effect of co-feeding of dietary molecules on the serum Ab levels. The results in Table 2.3 clearly show an augmented serum titer to LTB in the presence of Vit A, Vit B2, Vit D, Fructose, Mannose and Xylose. Little or no change with Vit B1, Vit B12, Vit C or Melibiose and almost complete inhibition with Vit B6, Vit E, Lactose, Melibiose and Sorbitol. The inhibition of an immune response seen with Vit B6, Lactose, Melibiose and Sorbitol is to be expected due to the similarities in structure of these compounds to Galactose which is claimed to be the specific sugar determinant on the GM1 ganglioside to which LTB is known to bind. These results are broadly suggestive that K99, 987P and LTB bind to and are internalized by discreet cells of the microvillous epithelium.

Dose response experiments (Tables 2.4, 2.5 and 2.6) demonstrate that is possible to stimulate the secretory arm of the immune system without concomitant stimulation of serum antibodies, or conversely to augment the serum response without affecting the level of secretory Abs, by the simple addition of dietary molecules to the orally presented mucosal immunogens. Thus cofeeding of large doses of Vit B12 or melibiose with K99 leads to a two to eightfold (respectively) increase in serum Ab with little concomitant increase in secretory

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Abs. Conversely cofeeding of Vit D in increasing doses lead to a drop in serum Abs and a rise in secretory Ab. Certain dietary molecules on the other hand also result in stimulation of both secretory and serum Ab titres as shown by an eightfold increase in serum Ab and a 1000 fold increase in S-IgA upon cofeeding of Vit C with 987P.

Experiments in which the mucosal immunogens were injected i.m.

together with vitamins or sugars showed little effect if any on the immune response thus demonstrating that the change in response due to cofeeding of these molecules with the mucosal immunogens must occur on or near the site of absorption of these molecules rather than directly upon the immune system (Table 2.7).

TABLE 2.1

Effects of dietary molecules on the immune response to orally administered K99 (20 μ g)*

Antibody Response

| <u>Dietary Molecule</u> | <u>Dose</u> | <u>Serum</u> | | <u>Intestinal</u> | |
|-------------------------|-------------|-----------------|---------------|-------------------|-----------------|
| | | <u>IgG</u> | <u>IgA</u> | <u>IgG</u> | <u>IgA</u> |
| none | - | 968 \pm 120 | <4 | 3.0 \pm 5.2 | 3.2 \pm 4.9 |
| Vit A | 20 μ g | 278 \pm 184 | 3.4 \pm 4.7 | 1.5 \pm 1.1 | 5.4 \pm 3.0 |
| Vit B1 | " | 117 \pm 107 | 1.5 \pm 2.0 | 2.7 \pm 1.0 | 2.1 \pm 0.9 |
| Vit B2 | " | 604 \pm 216 | <4 | 2.3 \pm 1.7 | 2.0 \pm 0.6 |
| Vit B6 | " | 14 \pm 50 | <4 | 2.0 \pm 1.5 | 3.5 \pm 8.2 |
| Vit B12** | " | 3377 \pm 1266 | 4.0 \pm 3.0 | <4 | <4 |
| Vit C** | " | 318 \pm 255 | 2.0 \pm 2.8 | 32 \pm 1.1 | 98 \pm 70 |
| Vit D** | " | 1921 \pm 640 | <4 | <4 | 6.3 \pm 2.7 |
| Vit E | " | 512 \pm 128 | <4 | <4 | 4.4 \pm 2.1 |
| Fructose | 50mM | 1782 \pm 966 | 8.4 \pm 3.7 | 2.9 \pm 1.6 | 34.7 \pm 14.2 |
| Lactose | " | 84 \pm 204 | <4 | <4 | 22.9 \pm 6.7 |
| Mannose | " | 1176 \pm 411 | 2.6 \pm 3.4 | 10.2 \pm 3.4 | 21.1 \pm 40.3 |
| Melibiose** | " | 1840 \pm 208 | 1.2 \pm 1.4 | 3.2 \pm 2.0 | 4.4 \pm 3.7 |
| Sorbitol | " | 77 \pm 179 | <4 | 1.3 \pm 0.4 | 20.5 \pm 3.4 |
| Xylose | " | 328 \pm 217 | <4 | 1.9 \pm 1.1 | 2.8 \pm 1.3 |

* The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 min at 37°C on day 21 after immunisation. Each value is the mean of five mice \pm 1 standard deviation.

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** Each value is the mean of 15 mice \pm standard deviation. These molecules were also tested in dose-response experiments (10-2000 μ g).

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Effects of dietary molecules on the immune response to oral 987P (20 μ g)*

| Dietary Molecule | Dose | Antibody Response | | | |
|---------------------|------------|-------------------|-----------------|-----------------|------------------|
| | | Serum | Intestinal | IgG | IgA |
| none | - | 776 \pm 64 | 10.8 \pm 8.8 | 10.9 \pm 1.7 | 48.5 \pm 1.8 |
| Vit A** | 20 μ g | 648 \pm 40 | 29.0 \pm 20.1 | 28.4 \pm 3.1 | 20.5 \pm 24.6 |
| Vit B1 | " | 891 \pm 127 | 12.1 \pm 3.6 | 9.4 \pm 1.5 | 14.7 \pm 5.5 |
| Vit B2 | " | 1082 \pm 271 | 31.1 \pm 11.9 | 17.8 \pm 7.6 | 21.1 \pm 11.9 |
| Vit B6 | " | 1782 \pm 509 | 23.6 \pm 8.7 | 32.8 \pm 9.3 | 39.9 \pm 7.1 |
| Vit B12 | " | 3983 \pm 1307 | 48.5 \pm 16.0 | 35.7 \pm 3.4 | 91.7 \pm 31.9 |
| Vit C** | " | 4521 \pm 1046 | 54.2 \pm 19.2 | 10.8 \pm 6.9 | 84.1 \pm 14.3 |
| Vit D** | " | 398 \pm 89 | 18.8 \pm 11.0 | 13.1 \pm 4.9 | 34.7 \pm 8.1 |
| Vit E | " | 3468 \pm 776 | 9.18 \pm 2.6 | 11.1 \pm 4.2 | 19.9 \pm 3.9 |
| Fructose | 50mM | 2048 \pm 894 | 10.8 \pm 3.6 | 15.1 \pm 4.7 | 23.5 \pm 3.2 |
| Lactose | " | 1128 \pm 662 | 15.3 \pm 2.9 | 19.6 \pm 2.5 | 21.7 \pm 6.1 |
| Mannose | " | 4970 \pm 2270 | 24.9 \pm 11.7 | 14.3 \pm 1.9 | 16.9 \pm 26.4 |
| Melibiose** | " | 1243 \pm 474 | 30.3 \pm 7.7 | 91.7 \pm 10.9 | 124.5 \pm 22.6 |
| Sorbitol** | " | 1389 \pm 307 | 38.8 \pm 19.1 | 22.9 \pm 4.6 | 91.7 \pm 18.6 |
| Xylose | " | 1024 \pm 941 | 6.2 \pm 1.9 | 19.6 \pm 7.1 | 21.4 \pm 5.4 |

* See Table 2.1

** See Table 2.1

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Effects of dietary molecules on the immune response to oral LTB4 (20 μ g)*

| | <u>Dietary Molecule</u> | <u>Dose</u> | <u>Antibody Response</u> | | | |
|----|-------------------------|-------------|--------------------------|------------|------------|-----------------|
| | | | <u>Serum IgG</u> | <u>IgA</u> | <u>IgG</u> | <u>IgA</u> |
| 5 | none | - | 1351 \pm 211 | <4 | <4 | 12.2 \pm 4.4 |
| | Vit A | 20 μ g | 4705 \pm 676 | <4 | <4 | 21.1 \pm 7.6 |
| 10 | Vit B1 | " | 1782 \pm 309 | <4 | <4 | 16.0 \pm 2.1 |
| | Vit B2 | " | 3565 \pm 908 | <4 | <4 | 16.0 \pm 3.7 |
| | Vit B6** | " | 9.18 \pm 1.11 | <4 | <4 | <4 |
| | Vit B12 | " | 1024 \pm 116 | <4 | <4 | 24.2 \pm 11.9 |
| | Vit C | " | 337 \pm 206 | <4 | <4 | 16.1 \pm 5.0 |
| | Vit D | " | 4097 \pm 74 | <4 | <4 | 13.9 \pm 2.2 |
| 15 | Vit E | " | 194 \pm 64 | <4 | <4 | 18.4 \pm 3.6 |
| | Fructose | 50mM | 6208 \pm 1192 | <4 | <4 | 10.5 \pm 3.3 |
| | Lactose** | " | 5.0 \pm 1.0 | <4 | <4 | <4 |
| | Mannose | " | 4096 \pm 658 | <4 | <4 | 24.2 \pm 8.1 |
| 20 | Melibiose | " | 512 \pm 76 | <4 | <4 | 8.0 \pm 4.0 |
| | Sorbitol** | " | 8.0 \pm 1.2 | <4 | <4 | <4 |
| | Xylose | " | 5404 \pm 2211 | <4 | <4 | 21.1 \pm 9.2 |

* See Table 2.1 for details of methodology.

** See Table 2.1 for details of methodology.

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TABLE 2.4

Effect of orally administered dietary molecules on the immune response to oral antigen (K99)*

| Dietary Molecule | Day 21 Immune response per dose (μg~, mM+) | | | | | | |
|-----------------------|--------------------------------------------|-----|------|------|------|-----|------|
| | 0.1 | 1.0 | 10 | 50 | 100 | 500 | 1000 |
| <u>Serum IgG</u> | | | | | | | |
| Vit B12 | 256 | 675 | 1176 | 1552 | 2352 | nd | 2352 |
| Vit C | 512 | 891 | 588 | 675 | 1024 | nd | 891 |
| Vit D | 588 | 588 | 776 | 891 | 1782 | nd | 891 |
| Melibiose | 128 | 256 | 675 | 4096 | 9410 | 256 | nd |
| <u>Serum IgA</u> | | | | | | | |
| Vit B12 | <4 | <4 | 4.0 | 5.2 | <4 | nd | <4 |
| Vit C | <4 | <4 | 4.0 | 16.0 | 16.0 | nd | 16.0 |
| Vit D | <4 | <4 | <4 | <4 | <4 | nd | <4 |
| Melibiose | <4 | <4 | <4 | 4.0 | 4.0 | 4.0 | nd |
| <u>Intestinal IgG</u> | | | | | | | |
| Vit B12 | <4 | <4 | <4 | <4 | <4 | nd | <4 |
| Vit C | <4 | 4.0 | 16.2 | 32.0 | 36.1 | nd | 38.0 |
| Vit D | <4 | <4 | <4 | <4 | <4 | nd | <4 |
| Melibiose | <4 | <4 | <4 | 4.0 | 4.0 | 4.5 | nd |
| <u>Intestinal IgA</u> | | | | | | | |
| Vit B12 | <4 | <4 | <4 | <4 | <4 | nd | 13.9 |
| Vit C | <4 | <4 | 4.0 | 16.0 | 36.7 | nd | 73.5 |
| Vit D | <4 | <4 | 4.0 | 8.0 | 13.9 | nd | 24.2 |
| Melibiose | 5.2 | 4.0 | 4.0 | 4.0 | 9.2 | 4.0 | nd |

~, dose in μg, vitamins; +, dose in mM, sugars.

* See Table 2.1

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TABLE 2.5

Effect of orally administered dietary molecules on the immune response to oral antigen (987P)

Day 21 Immune response per dose (ug~, mM+)

| 5 | Dietary molecule | 0.1 | 1.0 | 10 | 50 | 100 | 500 | 1000 |
|----------------|------------------|------|------|------|------|------|------|------|
| | | ug |
| Serum IgG | | | | | | | | |
| | Vit A | 512 | 675 | 388 | 891 | 675 | nd | 588 |
| 10 | Vit C | 891 | 1351 | 4096 | 8192 | 8192 | nd | 9410 |
| | Vit D | 2352 | 2048 | 256 | 73 | 147 | nd | 1351 |
| | Melibiose | 512 | 481 | 463 | 1250 | 1006 | 1292 | nd |
| | Sorbitol | 873 | 1133 | 1024 | 1400 | 2123 | 85 | nd |
| Serum IgA | | | | | | | | |
| 15 | Vit A | <4 | <4 | 16.0 | 30.0 | 36.0 | nd | 64.0 |
| | Vit C | 4.0 | 4.0 | 32.0 | 66.0 | 73.1 | nd | 86.7 |
| | Vit D | 64 | 16.0 | 16.0 | 16.0 | 28.0 | nd | 38.1 |
| 20 | Melibiose | nd |
| | Sorbitol | nd |
| Intestinal IgG | | | | | | | | |
| 25 | Vit A | <4 | <4 | 4.6 | 17.2 | 16.1 | nd | 16.0 |
| | Vit C | <4 | <4 | 16.0 | 6.0 | 5.2 | nd | 16.0 |
| | Vit D | <4 | <4 | <4 | 12.2 | 14.6 | nd | 14.0 |
| | Melibiose | 5.0 | 27 | 75 | 76 | 38.3 | 4.3 | nd |
| | Sorbitol | 50 | 74 | 64 | 93 | 294 | 257 | nd |
| Intestinal IgA | | | | | | | | |
| 30 | Vit A | <4 | <4 | 4.0 | 26.9 | 32.1 | nd | 16.0 |
| | Vit C | <4 | 4.6 | 55 | 337 | 1024 | nd | 776 |
| | Vit D | <4 | <4 | 16.0 | 147 | 25.2 | nd | 36.7 |
| | Melibiose | 7.6 | 39 | 65 | 159 | 58.0 | 16.0 | nd |
| | Sorbitol | 52 | 51 | 67 | 111 | 178 | 180 | nd |

~, dose in ug, vitamins; +, dose in mM, sugars

* see Table 2.1

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TABLE 2.6

Effect of orally administered dietary molecules on the immune response to oral antigen (LTB) ¹ (see Table 2.1 for antigen content)

| Dietary molecule | Day 21 Immune response per dose (ug~, mM+) | | | | | | Vit B6 10 500 | Sugars 10 1000 | IgG IgA |
|------------------|--------------------------------------------|--------|--------|--------|--------|-----|------------------|-------------------|---------|
| | 0.1 | 1.0 | 10 | 50 | 100 | 500 | | | |
| | ug~ | ug~ | ug~ | ug~ | ug~ | ug~ | | | |
| Serum IgG | | | | | | | | | |
| Vit B6 | 2352 | 5048 | 55.7 | 10.5 | 4.6 | nd | 4.0 | nd | (a) |
| Lactose | 381782 | 381782 | 381782 | 381782 | 381782 | 4.0 | nd | nd | nd |
| Sorbitol | 981024 | 981024 | 981024 | 981024 | 981024 | 4.0 | nd | nd | nd |
| Serum IgA | | | | | | | | | |
| Vit B6 | nd | <4 | <4 | <4 | <4 | nd | <4 | nd | nd |
| Lactose | <4 | <4 | <4 | <4 | <4 | <4 | <4 | nd | nd |
| Sorbitol | <4 | 1.4 | <4 | <4 | <4 | <4 | <4 | nd | (a) |
| Intestinal IgG | | | | | | | | | |
| Vit B6 | nd | <4 | <4 | <4 | <4 | nd | <4 | nd | nd |
| Lactose | <4 | <4 | <4 | <4 | <4 | <4 | <4 | nd | nd |
| Sorbitol | <4 | <4 | <4 | <4 | <4 | <4 | <4 | nd | nd |
| Intestinal IgA | | | | | | | | | |
| Vit B6 | 5.6 | <4 | <4 | <4 | <4 | nd | <4 | nd | nd |
| Lactose | 4.0 | 4.0 | <4 | <4 | <4 | <4 | <4 | nd | nd |
| Sorbitol | 4.0 | <4 | <4 | <4 | <4 | <4 | <4 | nd | nd |

~, dose in ug, vitamins; +, dose in mM, sugars

* see Table 2.1

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TABLE 2.7

Effect of varying the dose of co-administered dietary molecules on the immune response to intramuscularly presented antigen K99, 987P*

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Day 21 Immune response (U.S. vac)

| Antigen given | Dietary molecule | Dose (ug) | | | | | | Dose (mM) | Dose (mM) |
|---------------|------------------|-----------|------|------|------|------|------|-----------|-----------|
| | | 0.1 | 1.0 | 10 | 50 | 100 | 1000 | | |
| 10 (s) IgG | | | | | | | | | |
| K99 | Vit B12 | 1121 | 1468 | 1572 | 2328 | 4766 | 2109 | | |
| K99 | Vit D | 1024 | 1272 | 1168 | 1372 | 1489 | 1315 | | |
| 987P | Vit C | 1687 | 1529 | 1707 | 1700 | 1662 | 1891 | | |
| 15 | (mM) | 0.1 | 1.0 | 10 | 50 | 100 | 500 | | |
| (s) IgG | | | | | | | | | |
| K99 | Melibiose | 1024 | 1176 | 1057 | 1392 | 1262 | 989 | | |
| 987P | Melibiose | 1538 | 1622 | 1701 | 1519 | 1666 | 1621 | | |
| 20 987P | Sorbitol | 1670 | 1577 | 1548 | 1632 | 1711 | 1651 | | |

*. no serum IgA, intestinal IgG or intestinal IgA was detected

* See Table 2.1

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EXAMPLE 3

The two previous examples demonstrated that small doses of orally administered mucosal immunogens have the capacity to induce significant serum IgG titers with or without a parallel rise in secretory IgA antibody levels. Furthermore, it was shown that the immune response can be tailored by the co-administration of dietary molecules. The finding that the lectins used in the first study were capable of acting as carriers to prime for an anti-DNP response suggested that the potential exists for at least some of these mucosal immunogens to act as "carriers" for other antigens, therefore improving the relatively poor uptake of most antigens across the intestinal epithelium.

The following study was designed to investigate whether this carrier potential exists and to establish some of the various parameters for its successful use as a means of formulating new and more effective oral vaccines and/or orally administered drugs.

Materials and MethodsConjugation of antigens to the mucosal immunogensDinitrophenylation of carriers

DNFB was reacted with Lectins and carrier as described above (see general methods)

Lipopolysaccharide (LPS) and polysaccharide (PS) conjugation

S. typhimurium LPS and PS were purified as described previously (accompanying examples). LPS and PS were coupled to the MI using the periodate method (Avrameus and Ternynck, 1971).

Glutaraldehyde coupling

Leutinizing hormone releasing hormone (LHRH), bovine serum albumin (BSA) (from Sigma Chemical co. St. Louis, Miss) and *S. typhimurium* flagella were individually coupled to MI using the two step glutaraldehyde procedure of Ayrameus et al (1978). Briefly, the required protein was reacted with 0.2% glutaraldehyde for 2 hrs at R.T. proteins were dialysed overnight against carb/bicarb buffer pH 9.5 followed by the addition of MI at molar ratio's of 5, 10, 20 and 40:1, Antigen:MI, as required and reacted for 24 hrs at RT. Finally ethanclamine (Sigma) was added to a final concentration of 0.1M, (1 hr, RT) followed by overnight dialysis at 4C against 0.1 M carb/bicarb buffer pH 9.5.

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Peroxidase conjugated lectins

Commercial preparations of peroxidase conjugated to the lectins from Glycine max, Arachis hypogea, Tetragonolobus purpureas and conconavalin A were purchased from Sigma.

5 Chemical synthesis of LHRH conjugates

LHRH was conjugated to LTB using the glutaraldehyde procedure outlined above. Glutaraldehyde activated LHRH was added to LTB at a ratio of 20:1 LHRH:LTB and allowed to couple O/N at R.temp. The resultant conjugate was dialysed extensively against 0.1 M Carb/bicarb buffer pH 9.5 before feeding. Controls consisted of LHRH or LTB which had been treated with glutaraldehyde alone.

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TABLE 3.1

Antibody response to DNP-modified mucosal immunogens.

| Immunogen | Dose (ug) | Immune response* | | | |
|----------------|--------------|-----------------------|---------------------|---------------------------|-------------------------|
| | | Anti-DNP serum IgG | Anti-DNP Int IgA | Anti-Carrier serum IgG | Anti-Carrier Int IgA |
| K99 | 20 | <4 | <4 | 875±62 | 3.9±5.1 |
| K99 | 100 | <4 | <4 | 1351±128 | 16.7±2.3 |
| DNP6.K99 | 20 | 21±10.5 | <4 | 64±7.2 | <4 |
| DNP18.K99 | 500 | 1024±77 | 42±9.6 | 128±27.4 | 76±12.9 |
| DNP1.8.K99 | 500 | 1176±164 | 28±14.4 | 3565±192 | 88±21.0 |
| 987P | 20 | <4 | <4 | 891±76 | 27.8±13.6 |
| 987P | 100 | <4 | <4 | 1024±89 | 84±22.4 |
| DNP6.987P | 20 | 24±3.1 | <4 | 147±12.2 | <4 |
| DNP25.987P | 500 | 1024±244 | 14±3.1 | 111±34.1 | 68±19.2 |
| DNP2.5.987P | 500 | 1351±196 | 7±1.4 | 2048±166 | 128±38.4 |
| LTB | 20 | <4 | <4 | 1351±211 | 12.2±4.4 |
| DNP2.3.LTB | 20 | 24.3±5.6 | <4 | 445±35 | <4 |
| LTB | 100 | <4 | <4 | 891±56 | 4.0 |
| DNP6BSA | 20 | <4 | <4 | <4 | <4 |
| DNP6BSA | 100 | <4 | <4 | <4 | <4 |
| Con A** | 20 | 666±84 | <4 | nd | nd |
| PW-mitogen** | 20 | 641±119 | <4 | nd | nd |
| L. culinaris** | 20 | 954±48 | <4 | nd | nd |
| H. pomatia** | 20 | 591±127 | <4 | nd | nd |
| P. vulgaris** | 20 | 1378±110 | 4.8±2.3 | nd | nd |
| G. max** | 20 | 1529±65 | 3.1±6.9 | nd | nd |
| A. hypogea** | 20 | 1276±242 | <4 | nd | nd |
| U. europeus** | 20 | 1583±94 | <4 | nd | nd |

* The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 min at 37C. Each value represents the mean of 5 mice ±1 standard deviation.

** Each lectin was substituted with 4 DNP groups

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TABLE 3.2

Carrier potential of K99 for various antigens

| 5 | Immunogen Molar Dose | Antigen | Immune Response* | | | Carrier |
|----|----------------------|---------|------------------|-----------|-----------|---------|
| | | | Ratio (μg) | Serum IgG | Int IgA | |
| | | K99 | 20 | <4 | <4 | 875±62 |
| | | K99 | 100 | <4 | <4 | 1351±94 |
| 10 | BSA-K99 1:4 | 20 | <4 | <4 | <4 | <4 |
| | BSA-K99 1:5 | 500 | <4 | <4 | 73.5±11.2 | <4 |
| | BSA-K99 1:10 | 500 | <4 | <4 | 147±48.2 | <4 |
| | BSA-K99 1:20 | 500 | 222±47 | 126±29.2 | 3809±226 | 84±16.6 |
| | BSA-K99 1:40 | 500 | 73±19.6 | 44±7.5 | 3176±391 | 64±11.9 |
| 15 | Flag-K99 1:5 | 20 | <4 | <4 | <4 | <4 |
| | LPS-K99 1:1** | 20 | <4 | <4 | <4 | <4 |
| | PS-K99 1:1** | 20 | <4 | <4 | 229±101 | 5.2±2.6 |
| | BSA | 20 | <4 | <4 | - | - |
| | Flagellae | 20 | <4 | <4 | - | - |
| 20 | LPS | 20 | 12.1±2.7 | <4 | - | - |
| | PS | 20 | <4 | 4.2±0.4 | - | - |

* See Table 3.1

** Ratio based on weight.

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TABLE 3.3

Carrier potential of 987P for various antigens

Immune Response*

| 5 | | Antigen | | Carrier | | |
|-----------|----------|------------|----------------|----------------|----------------|-----------------|
| | Ratio | (μ g) | Serum IgG | Int. IgA | Serum IgG | Int. IgA |
| | | | | | | |
| 987P | - | 20 | <4 | <4 | 891 \pm 76 | 27.8 \pm 13.6 |
| 987P | - | 100 | <4 | <4 | 1024 \pm 83 | 84 \pm 26.7 |
| BSA-987P | 1:4 | 20 | <4 | <4 | 84 \pm 2.6 | <4 |
| 10 | BSA-987P | 1:5 | 500 | <4 | 256 \pm 49 | <4 |
| BSA-987P | 1:10 | 500 | 29.8 \pm 7.4 | 4.6 \pm 2.2 | 675 \pm 110 | <4 |
| BSA-987P | 1:20 | 500 | 306 \pm 88 | 122 \pm 47.6 | 4263 \pm 408 | 194 \pm 38.6 |
| BSA-987P | 1:40 | 500 | 124 \pm 47 | 110 \pm 38.4 | 4705 \pm 521 | 156 \pm 55 |
| Flag-987P | 1:5 | 20 | 1552 \pm 361 | <4 | - | - |
| 15 | LPS-987P | 1:1** | 20 | <4 | <4 | 194 \pm 28.6 |
| PS-987P | 1:1** | 20 | <4 | <4 | 337 \pm 96 | 5.2 \pm 6.8 |
| BSA | - | 20 | <4 | <4 | - | - |
| Flagellae | - | 20 | <4 | <4 | - | - |
| LPS | - | 20 | 12.1 \pm 2.7 | <4 | - | - |
| 20 | PS | - | 20 | <4 | 4.2 \pm 0.4 | - |

* See Table 3.1

** Ratio based on weight.

TABLE 3.4

Effects of altering dosage of substituted carrier on the immune

| 25 | Antigen | Dose (μ g) | Immune Response (Serum IgG)* |
|----|--------------|-----------------|------------------------------|
| | | Anti-BSA | Anti-Carrier |
| 30 | BSAO.03.K99 | 50.0 | 13.9 \pm 4.4 |
| | BSAO.03.K99 | 70 | 36.7 \pm 14.6 |
| | BSAO.03.K99 | 140 | 73.5 \pm 22.1 |
| | BSAO.03.K99 | 280 | 891 \pm 109 |
| 35 | BSAO.05.987P | 70 | 36 \pm 2.6 |
| | BSAO.05.987P | 140 | 168 \pm 23.7 |
| | BSAO.05.987P | 280 | 337 \pm 43.7 |
| | | | 1552 \pm 176 |
| | | | 2042 \pm 180 |

* See Table 2.1

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TABLE 3.5

Effect of chemically conjugated LHRH-LTB on reproductive organs of the female mouse*

| 5 | Conjugate | Coupling | Route | Dose | No. | Body weight (Mg) | Ovaries (Mg) | %Body wt | |
|-----------------|-----------|----------|-------|-------|-----|------------------|--------------|----------|---------------|
| | | | | | | | | uterus | ovario-uterus |
| +uterus O+U/tot | | | | | | | | | |
| | LHRH-LTB | | glut | os/os | 20 | 5 | 21.19 | 0.03 | 0.17% |
| | LHRH-LTB | | glut | os/os | 50 | 5 | 21.16 | 0.04 | 0.23% |
| 10 | LHRH | | glut | os/os | 50 | 6 | 20.13 | 0.11 | 0.62% |
| | LTB | | glut | os/os | 50 | 3 | 18.18 | 0.14 | 0.76% |
| | LHRH-LTB | | glut | im/im | 50 | 6 | 20.16 | 0.16 | 0.97% |
| | LHRH,LTB | | mix | os/os | 50 | 7 | 27.32 | 0.15 | 0.55% |
| | LHRH,LTB | | mix | im/im | 50 | 7 | 30.07 | 0.26 | 0.73% |
| 15 | LTB | | glut | os/os | 50 | 7 | 28.26 | 0.24 | 0.87% |
| | - | - | glut | os/os | - | 5 | 19.19 | 0.16 | 0.82% |

* Animals received antigen on days 0 and 14. On day 28 mice were euthanased and their reproductive tracts weighed.

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TABLE 3.6

Effect of genetically constructed LHRH conjugates on the reproductive organs of the female mouse

| 25 | Conjugate | Route | Dose | Body weight (Mg) | Ovaries | %Body wt | |
|----|-----------------|-------|------|------------------|---------|----------|----------|
| | | | | | | +uterus | O+U/tot* |
| | B-gal-(LHRH)3.5 | im/im | 20 | 19.47 | 0.10 | 0.49 | |
| | B-gal-(LHRH)3.5 | im/im | 20 | 18.44 | 0.10 | 0.56 | |
| | B-gal-(LHRH)3.5 | im/im | 50 | 19.39 | 0.10 | 0.54 | |
| | LTB-(LHRH)3.5 | os/os | 20 | 19.35 | 0.08 | 0.39 | |
| 30 | LHRH | os/os | 20 | 19.34 | 0.10 | 0.44 | |
| | LHRH | im/im | 20 | 19.39 | 0.15 | 0.76 | |
| | - | im/im | - | 17.74 | 0.08 | 0.46 | |
| | - | os/os | - | 18.63 | 0.11 | 0.60 | |

35 For all intramuscular injections antigens were administered in montanide. Im/im controls received montanide/saline mixtures.

* Animals received antigen on days 0 and 14. On day 28 mice were euthanased and their reproductive tracts weighed.

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Genetic fusion of LHRH to β -galactosidase and LTB
CONSTRUCTION OF A PLASMID VECTOR
EXPRESSING A FUSED LTB/LHRH HYBRID POLYPEPTIDE

1. DNA Fragment Containing LTB Coding Sequences

A Hind III fragment was obtained from a pBR322-based plasmid containing the cloned LTB gene (Leong *et al.*, 1985), from the plasmid NP307 in *E. coli* strain RC411 (Dallus *et al.*, 1979) which had been modified, using a Spe I site near the stop codon for LTB, and then mung bean nuclease digestion, using standard conditions. (Unless specified otherwise, the conditions used for standard recombinant DNA techniques and nucleic acid modifying enzymes are as described in Molecular Cloning, A Laboratory Manual, Maniatis, Fritsch and Sambrook. Cold Spring Harbor Laboratory, 1982). This eliminated the stop codon normally found after amino acid 123 in LTB, removing 9 base pairs of DNA and fortuitously generating a Hind III site as shown below:

H III
 ↓ Ile Ser Met Lys ↓
LTB coding sequence ATC AGT ATG AAA GCT
121 122
 Size = 573 bp

This fragment was ligated into the vector pUC13 (Messing, 1983) after Hind III digestion and phosphatase treatment of the plasmid using standard conditions. This served to place the remaining polylinker region of pUC13, including a PstI, SalI, XbaI, BamHI, SmaI, SstI and EcoRI sites downstream of the LTB sequence containing DNA insert.

2. Creation of Synthetic LHRH Coding Oligonucleotides

Two oligonucleotides, of 30 bases in length, with sequences described in A and B below, were designed to form overlapping hybrid duplexes, as shown in C, which result in a duplex which will encode linear end-to-end repeats of the 10 amino acids encoding the peptide hormone LHRH (Schally and Coy, 1983). Role of Peptides and Proteins in Control of Reproduction, McCann and Dhindsa eds, Elsevier Science Publishing Co, Inc. pp 89-110). In this sequence, glutamic acid replaces the normal N-terminal pyroglutamic acid.

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A. 5' GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG 3'
B. 5' GTA GGA CCA GTG CTG CCC GGG TCG AAG GCC 3'
C. A A A

5 The oligonucleotides were annealed together for 1 hr at 40°C
in 50 mM NaCl, 10 mM Tris pH 7.5, end filled with Klenow, and then the
mixture was ligated into SmaI cut M13 mp18, using standard
procedures. M13 phage containing inserts were isolated, and the DNA
sequences of the inserts were determined by the dideoxy technique.

10 One recombinant, designated as P29, was chosen for the fused construct. Its DNA sequence, together with the amino acids it encodes, in the region of the insert at the *Sma*I site is given below.

N terminus of β -galactosidase, α fragment

Met Thr Met Ile Thr Asn Ser Ser Ser Val Pro Leu Arg

ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC CTT CGA

EcoRI

Pro Gly Glu His Trp Ser Tyr Gly Leu Arg Pro Gly

CCC GGG GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG

CII His Trp Ser Tyr Gly Leu Arg Pro Gly Glu His Trp

GAG CAC TGG TCC TAC GGC CTT CGA CCC GGG GAG CAC TGG

← 15. **Signs** **Age** **Pre** **Clu** **Clu** **Age** **Pre** **Low** **Clu** **See** **Ths**

Ser Tyr Gly Leu Arg Pro Gly Gly Asp Ile Gln Gln Asp

III TAGGGG CTT TGA CCC GGG GGG GAT CCTT GAT GAG TGG

3. 人體的形狀 一、形狀 二、形狀 三、形狀

The replicative form DNA of P29 was digested with EcoRI and HincII (sites indicated in DNA sequence above), and end filled using standard conditions. The small 140 base pair fragment was isolated from a polyacrylamide gel.

3. Construction of the LTB/LHRH Fusion Vector

The pUC13 plasmid containing the LTB coding sequence inserted in

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the HindIII site (section 1 above) was digested with SalI, end filled and phosphatased using standard conditions. The vector DNA was then ligated to the end filled EcoRI/HincII fragment from P29 (Section 2 above). The fusion should have the amino acid sequence below.

5 aal - 122 (lys)-ala-trp-ala-ala-gly-arg-asn-ser-ser-ser-val- pro-
LTB coding sequence.

leu-arg-pro-gly-[glu-his-trp-ser-tyr-gly-leu-arg-pro-gly]₃-

LHRH

10 gly-asp-pro-leu-glu-ser-arg-leu

There is a stop codon 24 bases beyond the LHRH sequence which defines the production of a 176 amino acid polypeptide, with 122 amino acids from LTB, 34 amino acids encoding the LHRH repeat, and an additional 20 amino acids derived from the adjoining regions.

15 The correct construct was screened by digesting DNA from minipreps with Eco RI and looking for plasmids with the appropriately larger Eco RI fragment. The putative positives were further screened for the expression of this polypeptide, driven from the lac promoter of pUC13. Bacterial extracts were analysed on polyacrylamide gels followed by transfer to nitrocellulose paper and Western blotting with rabbit antisera directed against an LTB and LHRH conjugate. A peptide of the expected size was detected by both antisera.

20 4. Construction of expression plasmid K66 and expression strain BTA1185

25 A 573 bp Eco RI fragment of the pUC13 LTB-LHRH fusion plasmid described in 3, which contains the LTB-LHRH fusion coding region in its entirety, was isolated from an agarose gel and ligated into Eco RI cut, phosphatased expression vector pKK223-3 (from Pharmacia). The resultant expression plasmid PBTA K66, placed the expression of the fusion protein under the control of the tac promoter, where expression is induced with IPTG. The plasmid was transformed into *E. coli* host strain JM101 (SupE, thi, (lac-pro AB) [F' traD36, pro AB lacI^q Z M 15] to give the host vector expression system BTA1185.

30 5. Production and Purification of LTB/LHRH Fusion Protein for Animal Trials.

The LTB(LHRH) 3.5 producing strain was grown as described

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5 ability of the cells to synthesize the enzyme (LacZ) could be monitored and previously. After induction with IPTG for 2 h bacteria were pelleted by centrifugation (3,000 x g, 10 min at 4°C). Bacteria were then resuspended in dH₂O and lysed in a French Press. After removal of the bacterial debris by centrifugation (18,000 x g, 10 min, at 4°C) the supernatant was loaded onto an agarose-galactose column (Sigma). The fusion protein was then eluted with 0.5M galactose and dialysed against 0.1M carb/bicarb buffer pH 9.5.

Antigen administration and measurement of the immune response

10 All oral presentation procedures, antibody collections and ELISA determinations were as described previously.

RESULTS

Demonstration of the carrier potential of the mucosal immunogens

15 All of the mucosal immunogens tested showed the capacity to effectively transport the covalently attached hapten DNP across the intestinal mucosa and to elicit a serum anti-DNP Ab response after feeding of the dinitrophenylated-MI. DNP-modified BSA, however was completely ineffective in eliciting an anti-DNP or anti-BSA response when fed at the concentrations tested (Table 3.1). Initial experiments in which K99 and 987P were complexed to much larger 20 molecules than DNP were unsuccessful in generating immune responses to either the mucosal immunogen or to the molecule coupled to it (Tables 3.2 & 3.2), possibly due to steric interference in the binding of the pili to the mucosal epithelium. It was therefore decided to vary the ratio of antigen to MI. When various ratios of BSA:pili were tested, 25 it was found that when ratios of greater than 1:20 BSA:pili were fed it was not possible to generate either anti-BSA or anti-pili responses even with a dose of 500 µg, demonstrating that it was not possible for the complexes to effectively associate with the mucosal epithelium and to therefore generate an immune response. However when ratios of 30 1:20 or 1:40 were employed good responses to both BSA and to pili were observed (Tables 3.2 & 3.3). The magnitude of the immune response was readily varied by altering the doses of complex fed (Table 3.4).

35 Oral administration of LHRH coupled to LTB lead to a significant reduction in the combined uterine and ovarian weights of female mice receiving either 20 or 50 µg LHRH-LTB (P < 0.05) (Table 3.5,3.6). No such weight loss was seen with either LHRH or LTB fed alone or together or to intramuscular injection of LHRH-B-galactosidase,

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LHRH-LTB, or free LHRH. The effect of the weight loss was also seen developmentally as there was a complete absence of mature follicles in the ovaries, thus, the animals were effectively "castrated". There was a slight reduction in reproductive tract weights when mice were fed the genetically constructed LTB-(LHRH)_{3.5} fusion protein (Table 3.6) but in this experiment, the reduction was not significant at the doses tried.

EXAMPLE 4

Induction of cell-mediated immunity after oral administration of antigen

Feeding of mucosal immunogens was shown to be effective in eliciting humoral responses as measured by the production of serum and intestinal antibodies. It was not known, however, whether there was a concomitant stimulation of a cell mediated immune (CMI) response to the mucosal immunogens.

The following study was designed to compare the CMI generated by oral presentation of a mucosal immunogen with that generated by classic subcutaneous (s.c.) injection of antigen in Complete Freund's Adjuvant (CFA).

Methods

Male C57BL/6J mice were immunized by feeding 20 µg antigen in 0.1 M carb/bicarb buffer pH 9.65 or by injecting 20 µg antigen in CFA s.c. Controls received only buffer or adjuvant. Seven days after immunization mice were injected in the left back footpad with 10 µg of antigen in 20 µl saline, and injected with 20 µl saline alone in the right rear footpad. After a further 24 hours, the difference in thickness between the left and right rear footpads was measured using a micrometer.

Results

The results shown in Table 4.1 demonstrates that a good cellular immune response is generated upon oral feeding of either 987P or LTB mucosal immunogens. In fact the response was surprisingly high as it was only slightly smaller than that generated by the s.c. injection of these antigens in CFA.

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Table 4.1 *Generation of a cell mediated immune response upon oral administration of various forms of mucosal immunogens*

| 5 | Antigen | Administration route | Immunisation route | |
|---|---------|----------------------|--------------------|--------------|
| | | | Oral | Subcutaneous |
| | 987P | | 11 ± 2 | 31 ± 6.6 |
| | LTB | | 5 ± 4.5 | 14 ± 3.7 |

* Results represent the increase in footpad size after immunization with antigen. Results are the mean of 5 mice ± standard deviation.

INDUSTRIAL APPLICABILITY

Industrial applications of the invention include the preparation of oral medicaments for administration to vertebrate hosts.

Potential Vaccine Candidates for Oral Vaccine

Allergens: Various Grass Pollens: barley, couch,

Weed Pollens: clover, dock

Tree Pollens: ash, cypress

Plant Pollens: broom

Epithelia: cat hair, dog hair, pig hair

Miscellaneous: house dust, wheat chaff, Kapok.

Hormones: LHRH, FSH, HGH, Inhibin

Vaccines: Haemagglutinins from

Influenza, Measles, Rubella, Smallpox, Yellow

Fever, Diphtheria, Tetanus, Cholera, Plague,

Typhus, BCG, Haemophilus influenzae, Neisseria

catarrhalis, Klebsiella pneumoniae,

Pneumococci, streptococci esp. S. mutans

E. coli, N. gonorrhoeae, N. meningitis, N.

catarrhalis, Yersinia spp., Pseudomonas

aeruginosa, Pseudomonas spp., Moraxella bovis,

Bacteroides nodosus, Staph. spp., Strep. spp.,

Bordetella spp.

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A party to a copending case has filed CLAIMS which read as follows:

1. A complex comprising: an immunogen linked to a carrier molecule which carrier molecule is capable of specifically interacting with the mucosal epithelium of a vertebrate host; wherein both the immunological activity of the immunogen and the capacity of the carrier molecule to specifically interact with the mucosal epithelium of the vertebrate host is substantially maintained, and said complex is capable of eliciting a systemic, cellular and/or mucosal immune response in the vertebrate host.
2. A complex according to claim 1 wherein said immunogen is selected from; all, part(s), an analogue, homologue, derivative or combination thereof of a hormone, therapeutic agent, antigen or hapten.
3. A complex according to claim 1 wherein said complex is capable of eliciting a systemic or cellular immune response in the vertebrate host.
4. A complex according to claim 1 wherein said complex is capable of eliciting a mucosal immune response in the vertebrate host.
5. A complex according to any one of claims 1 to 4 wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of an antigen or hapten.
6. A complex according to any one of claims 1 to 5, wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of a hormone, e.g. gonadotropin, luteinizing hormone, follicle stimulating hormone, thyrotropin, adrenocorticotropin, prolactin, etc.
7. A complex according to any one of claims 1 to 6 wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of a hormone releasing hormone.
8. A complex according to any one of claims 1 to 6 wherein the immunogen is FSH, HGH, inhibin, etc.
9. A complex according to any one of claims 1 to 5, wherein the immunogen is an allergen, e.g. a pathogen or a food.
10. A complex according to claim 9, wherein the allergen is a grass pollen, weed pollen, tree pollen, plant pollen, cat hair, dog hair, pig hair or other epithelial or house, dust, wheat chaff or kapok.
11. A complex according to any one of claims 1 to 4, wherein the immunogen is a surface protein derived from influenza, measles, Rubella, smallpox, yellow fever, diphtheria, tetanus, cholera, plague, Typhus or BCG causing agents, Haemophilus influenzae, Neisseria.

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catarrhalis, Klebsiella pneumoniae, pneumococci and streptococci; or a pilus derived from E.coli, N.gonorrhoeae, N.neningitidis, N.catarrhalis, Yersinia spp., Pseudomonas aeruginosa, Pseudomonas spp., Moraxella bovis, Bacteroides nodosus, Staphylococci spp and Bordetella spp; and two antigenic and two in type bacterial

12. A complex according to any one of claims 1 to 4, wherein either the immunogen is a surface polysaccharide derived from diphtheria, tetanus, cholera, plague, Typhus or BCG causing agents, Haemophilus influenzae, Neisseria catarrhalis, Klebsiella pneumoniae, pneumococci and streptococci; or a pilus derived from E.coli, N.gonorrhoeae, N.neningitidis, N.catarrhalis, Yersinia spp., Pseudomonas aeruginosa, Pseudomonas spp, Moraxella bovis, Bacteroides nodosus, Staphylococci spp, Streptococci spp and Bordetella spp.

13. A complex according to any one of claims 1 to 4, wherein the immunogen is a secretory product derived from diphtheria, tetanus, cholera, plague, Typhus or BCG causing agents, Haemophilus influenzae, Neisseria catarrhalis, Klebsiella pneumoniae, pneumococci and streptococci; or a pilus derived from E.coli, N.gonorrhoeae, N.neningitidis, N.catarrhalis, Yersinia spp., Pseudomonas aeruginosa, Pseudomonas spp, Moraxella bovis, Bacteroides nodosus, Staphylococci spp, Streptococci spp and Bordetella spp.

14. A complex according to any one of claims 11 to 13, wherein the surface protein, polysaccharide or secretory product is derived from Streptococcus mutans or one or more of enterococci subsp.

15. A complex according to any one of claims 1 to 14 wherein the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of a bacterial adhesin, viral haemagglutinin, a toxin or the binding subunit thereof, or a lectin whether of plant or other origin.

16. A complex according to claim 15 wherein the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of the heat-labile toxin of enterotoxigenic E.coli.

17. A complex according to claim 15 wherein the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of a bacterial pilus.

18. A complex according to claim 17 wherein the bacterial pilus is the K99 or 987P pilus of E.coli.

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19. A complex according to claim 17 wherein the bacterial pilus is CFAI, CFAII, K88 or F41.

20. A complex according to claim 15 wherein the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of a lectin.

21. A complex according to claim 20 wherein the lectin is Concanavlin A, Pokeweed mitogen or the lectin from Lens culinaris, Helix pomatia, Glycine max, Arachis hypogaea, or Ulex europeus.

22. A complex according to claim 20 wherein the lectin is Abrin, Asparagus pea, Broad bean, Camels foot tree, Castor bean, Fava bean, Green marine algae, Hairy vetch, Horsegram, Horseshoe crab, Jack bean, Japanese wisteria, Jequirity bean, Scotch Laburnum, Lima bean, Limulin, Lotus, European Mistletoe, Mung bean, Osage orange, Pogada tree, Garden pea, Potato, Red kidney bean, Red marine algae, Siberian pea tree, edible snail, garden snail, Spindle tree, Sweet pea, Tomato, Wheat germ or Winged pea lectin.

23. A complex according to claim 15, wherein the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of a viral haemagglutinin.

24. A complex according to claim 23 wherein the viral haemagglutinin is a haemagglutinin derived from influenza, measles, Rubella, smallpox or yellow fever virus.

25. A complex according to any one of claims 1 to 4 wherein the immunogen is all, part, an analogue, homologue, derivative or combination thereof of LHRH and the carrier molecule is all, part, an analogue, homologue, derivative or combination thereof of LTB.

26. A process for the production of a complex according to any one of claims 1 to 25 which process comprises:

- reacting the immunogen with the carrier molecule to form said complex;
- chemically modifying the immunogen to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex; or
- chemically modifying the carrier molecule to provide at least one functional group capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;
- chemically modifying the immunogen and the carrier molecule

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to provide functional groups capable of forming a chemical linkage, and reacting the immunogen and carrier molecule to form said complex;

(e) reacting the immunogen with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex;

(f) reacting the carrier molecule with at least one linking agent and reacting the immunogen and the carrier molecule to form said complex;

(g) reacting the immunogen and the carrier molecule with at least one linking agent, and reacting the immunogen and the carrier molecule to form said complex; or

(h) a combination of any of the preceding process steps.

27. A process for the production of a complex according to any one of claims 1 to 25 which process comprises:

providing a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA; transforming a host with said recombinant DNA molecule so that said host is capable of expressing a hybrid, proteinaceous product which comprises said complex; culturing said host to obtain said expression; and collecting said hybrid proteinaceous product.

28. A process for the production of a complex according to any one of claims 1 to 25 which process comprises:

(a) chemically synthesizing the immunogen and/or the carrier molecule, and forming the complex by reactions according to the process of claim 26;

or (b) synthesizing a hybrid peptide comprising amino acid sequences of the immunogen and the carrier molecule.

29. A process according to claim 28 wherein the peptide is prepared by solid phase, enzymatic or manual peptide synthesis.

30. A process according to claim 29 wherein the peptide is prepared by solid phase peptide synthesis.

31. A process according to claim 30 wherein the synthesized immunogen or carrier molecule whilst bound to the resin of the solid phase peptide synthesizer is coupled to the carrier molecule or immunogen respectively.

32. A process according to any one of claims 28 to 31 wherein

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the synthetic peptide is all, part, an analogue, homologue, derivative or combination thereof of LHRH.

33. A process according to claim 26 wherein the carrier molecule is LTB and the linking agent is glutaraldehyde.

34. A complex according to any one of claims 1 to 25 when made by a process according to any one of claims 26 to 31.

35. An expression product of a transformant host which comprises a complex according to any one of claims 1 to 25.

36. A transformant host transformed with a recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequences of all, part, an analogue, homologue, derivative or combination thereof, of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of all, part, an analogue, homologue, derivative or combination thereof of the carrier molecule, and vector DNA.

37. A transformant host according to claim 36 wherein said host is a Gram negative bacterium, a Gram positive bacterium, a yeast, fungus, or a higher eukaryotic cell.

38. A transformant host according to claim 36 or claim 37 wherein said host is E.coli.

39. A culture of the transformant microorganism designated: ATCC

40. A recombinant DNA molecule comprising a first DNA sequence which on expression codes for the amino acid sequence of the immunogen, a second DNA sequence which on expression codes for the amino acid sequence of the carrier molecule, and vector DNA.

41. A recombinant DNA molecule according to claim 40 wherein said vector DNA is plasmid DNA.

42. Plasmid pBTAK66.

43. A recombinant DNA molecule according to claim 40 wherein said vector DNA is viral, bacteriophage or cosmid DNA.

44. A polynucleotide sequence which comprises a first hybrid polynucleotide sequence which acts as a coding sequence for a fusion product comprising an amino acid sequence of an immunogen fused to an amino acid sequence of a carrier molecule, a polynucleotide sequence sufficiently related to said first hybrid polynucleotide sequence so as to hybridize to said first hybrid polynucleotide sequence, a polynucleotide sequence related by mutation, including single or

multiple base substitutions, deletions, insertions and inversions, to said first hybrid polynucleotide sequence or hybridizing sequence or a polynucleotide sequence which on expression codes for a polypeptide which displays similar biological or immunological activity to said fusion product.

45. A polynucleotide sequence according to claim 44 wherein the first hybrid polynucleotide sequence acts as a coding sequence for the amino acid sequence of all, part, an analogue, homologue, derivative or combinations thereof, of an antigen or hapten fused to the amino acid sequence of a carrier molecule.

46. A polynucleotide sequence according to claim 44 wherein the first hybrid polynucleotide sequence acts as a coding sequence for the amino acid sequence of all, part, an analogue, homologue, derivative or combinations thereof of LHRH fused to the amino acid sequence of a carrier molecule.

47. A medicament which comprises a complex according to any one of claims 1 to 25 together with a pharmaceutically acceptable carrier or diluent.

48. A medicament according to claim 47 adapted for oral administration.

49. A medicament according to claim 47 adapted for nasal administration.

50. A medicament according to any one of claims 47 to 49 wherein said medicament is in capsule, tablet, slow release, elixir, gel, paste or nasal spray form.

51. A medicament according to any one of claims 47 to 50 which additionally comprises a dietary molecule which dietary molecule can selectively modulate the magnitude and/or type of immune response to the immunogen of the medicament.

52. A medicament according to claim 51 wherein the dietary molecule is selected from amino acids, vitamins, monosaccharides and oligosaccharides.

53. A medicament according to claim 51 or claim 52 wherein the dietary molecule is selected from vitamin A, vitamin B₁, vitamin B₂, Vitamin B₆, Vitamin B₁₂, Vitamin C, Vitamin D, Vitamin E, fructose, lactose, mannose, melibiose, sorbitol or xylose.

54. A method of presenting a complex according to any one of

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claims 1 to 25 to a vertebrate host which method comprises the mucosal administration of a medicament according to any one of claims 47 to 51.

55. A method of presenting a complex according to any one of claims 1 to 25 to a vertebrate host which method comprises the oral administration of a medicament according to any one of claims 47, 48, 50 and 51.

56. A method of presenting a complex according to any one of claims 1 to 25 to a vertebrate host which method comprises the nasal administration of a medicament according to any one of claims 47, 49, 50 or 51.

57. A method of inhibiting the gonadal function in a mammal, which method comprises the mucosal administration of a medicament according to any one of claims 47 to 51.

58. A method of selectively modulating the magnitude and/or type of immune response to the immunogen of a complex according to any one of claims 1 to 25 which method comprises the mucosal administration of a medicament according to claim 51 to a host vertebrate.

59. A method of selectively modulating cellular immune responses to a complex according to any one of claims 1 to 25 which method comprises the mucosal administration of a medicament according to claim 45 in such a way as to specifically increase or decrease the cellular immune response to the complex in the vertebrate host.

60. A method according to claim 58 or claim 59 which comprises the administration of a medicament according to any one of claims 47 to 50 and a dietary molecule.

61. A complex substantially as hereinbefore described with reference to the Examples.

62. A method for the induction or modulation of an immune response in a host substantially as hereinbefore described with reference to the Examples.

63. A carrier molecule substantially as hereinbefore described with reference to the Figure.

Sequence 111 contains 15amino acids (res 10-24) of 21amino acids
Type 1 Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-Met-Phe-Lys-Gly-
K88 Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-
K99 Asn-Thr-Gly-The-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-
987P Ala-Pro-Val-Glu-Asn-Asn-Thr-Cys-Gln-Ala-Asn-Leu-Asp-Phe-
Neisseria Phe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Ile-Ala-Ile-Val-Gly-
15 20 25

Type 1 Glu-Val-Val-Asn-Ala-Ala-
K88 Ser-Ile-Thr-Ala-Asp-Asp-Tyr-Arg-
K99 Thr-Cys-Thr-Ile-Glu-Pro-Glu-Ala-
987P Thr-Gly-Lys-Val-Thr-Ala-x-Leu-
Neisseria Ile-Leu-Ala-Ala-Val-Ala-Leu-Pro-

Fig.1 N-terminal amino acid sequence of the 987P pilin subunit. The N-terminal sequences of other pilin proteins are given for comparison.

LODGED AT SUB OFFICE

21 MAY 1986

Sydney

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 86/00135

I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. 4 A61K 39/385, C07K 17/00, C12N 1/20, 15/00

II. FIELDS SEARCHED

| Minimum Documentation Searched | |
|-----------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|
| Classification System | Classification Groups |
| IPC: A61K 39/385, 39/39, 39/44, 47/00, C07K 7/00, 17/00, C07K 7/20, 13/00, C12N 1/20, 15/00, C07C 103/52 | Chemical Abstracts keywords:pili, Fimbriae, vaccine |
| Documentation Searched other than Minimum Documentation and to the Extent that such Documents are Included in the Fields Searched | |

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Character of Document (with indication where disclosure of the relevant passages) | Relevant Classification |
|----------|-----------------------------------------------------------------------------------------------|----------------------------------------------------------|
| X | US, A, 4 496 538 (L.K. LANCE) 29 January 1985 (29.01.85) | 1-5, 11, 12, 15, 26, 28, 47 |
| X,P | AU, A, 44588/85 (PRAXIS BIOLOGICS INC) 13 February 1986 (13.02.86) see pages 8, 9, 14, 26, 27 | 1,3-5, 9, 11, 12, 13, 15-19, 26, 33-34, 47 |
| X,P | WO, A, 85/05037 (SYNTEK) 21 November 1985 (21.11.85) | 1-5, 11, 17-19 26, 27, 35-38 40, 41, 43-45 47-50, 55, 56 |
| X,P | WO, A, 85/04654 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 24 October 1985 (24.10.85) | 1,3-5, 11, 15, 17, 26-31, 34-38, 40-41 43-50, 54-56 |
| X | WO, A, 83/03971 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 24 November 1983 (24.11.83) | 1-5, 11-13, 15 26, 27, 35-38, 40, 41, 43-45 47 |

continued

IV. SPECIAL CATEGORIES OF CITED DOCUMENTS

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" document or document but published on or after the international filing date
- "C" document which may throw doubt on priority, claim(s) or which is cited to assist in the publication or sale of another's claim or other document (e.g. a specification)
- "D" document referred to in an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the document being considered, but which may be of interest due to its disclosure of a particular feature

"X" document of particular relevance, the disclosure of which cannot be considered, due to or cannot be considered, prior to or after the filing date

"Y" document of particular relevance, the disclosure of which cannot be considered to relate to a particular area of the document, e.g. combination with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

13 August 1986 (13.08.86)

Date of Mailing of this International Search Report

26 AUGUST 1986 (26.08.86)

International Searching Authority

AUSTRALIAN PATENT OFFICE

Signature of Authorized Officer

J.H. CHAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|
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| X | US, A, 4372883 (MATUHASHI et al) 8 February 1983 (08.02.83) | 1-6 |
| X | Journal of Immunological Methods, Volume 48 issued 1982 (Amsterdam, Netherlands) P.R. Lambden and J.E. Heckels, 'Synthesis of Immunogenic oligosaccharide-Protein Conjugates from Lipopolysaccharides of <u>Neisseria gonorrhoeae</u> P 9', see pages 233-240 | 1-5, 1, 12, 15, 17, 26, 47 |
| X | EP, A, 0 116 201 (CHIRON CORP) 22 August 1984 (22.08.84) see page 2 line 11 - page 3 line 16 | 40, 41, 43 44 |
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| X | GB, A, 2071671 (HARVARD COLLEGE PRESIDENT AND FELLOWS OF CAMBRIDGE) 23 September 1981 (23.09.81) | 40, 41, 43 44 |
| X | EP, A, 0 020 147 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 10 December 1980 (10.12.80) see page 2 line 5 - page 3 line 6, page 11 line 36 - page 12 | 40, 41, 43, 44 |
| X | EP, A, 0 049 945 (BACTEX INC) 21 April 1982 (21.04.82) | 63 |
| X | GB, A, 2 094 314 (THE WELLCOME FOUNDATION LTD) 15 September 1982 (15.09.82) | 63 |
| X | GB, B, 1 529 871 (DORNER et al) 25 October 1978 (25.10.78) | 63 |
| Y | AU, A, 34497/84 (AKZO N.V.) 26 April 1985 (26.04.85) See pages 2-9 | 1-3, 5-7, 11-12, 15, 25-26, 28, 32, 34, 48- 50 |
| Y | US, A, 4556555 (K.L. ESBENSHADE) 3 December 1985 (03.12.85) | 7, 25, 57 |
| Y | AU, A, 11990/83 (CYTOGEN CORPORATION) 15 September 1983 (15.09.83) | 26 |
| Y | Progress in Allergy, Volume 33, issued 1983 (Basel, Switzerland) Schoolnik et al, 'A Pilus Peptide Vaccine for the Prevention of Gonorrhoea', see pages 314-331 | 1-63 |
| X, Y | M. Sussman, 'The Virulence of Escherichia coli' 1985, Academic Press, see pages 82-37, 111-119 | 63 |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT, ON
INTERNATIONAL APPLICATION NO. PCT/AU 86/00135**

NO FURTHER WORK IS BEING DONE ON THIS APPLICATION

CONTINUATION BEING MADE ON THE INTERNATIONAL SEARCH REPORT

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Members | | | | | |
|----------------------------------------------|----------|-----------------------------------------------------|----------------------|-----------------------------------------|----------------------|-------------------------------------------|--|
| US | 4496538 | AU 18227/83 ES 523905 ZA 8304939 | DK FI | 935/84 840874 | EP NO | 98581 840818 | |
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| WO | 8505037 | AU 42994/85 | | | | | |
| WO | 8504654 | AU 41590/85 | NO | 854903 | ZA | 8502629 | |
| WO | 8303971 | AU 17062/83 FI 840090 | EP NO | 108146 840089 | ES NZ | 522315 204229 | |
| AU | 14705/76 | BE 842814 DK 2630/76 GB 1553664 NL 7606379 | CA ES IT US | 1061252 448827 1078623 4136167 | DE FR JP ZA | 2626530 2313942 51151322 7603370 | |
| US | 4372883 | CA 1156553 GB 2061955 | DE | 3032488 | FR | 2464074 | |
| EP | 116201 | DK 5778/83 | JP | 59132892 | | | |

NO 1170130 NOVAKA JAROMÍR PAVEL ALFRED
 RESEARCH LABORATORY FOR POLYMERIC MATERIALS
 ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
 INTERNATIONAL APPLICATION NO. PCT/AU 86/00135 (CONTINUED)

Patent Document
 Cited in Search
 Report

Patent Family Members

| | | | | |
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| GB | 2071671 | AT 1222/81 DE 3110031 FR 2478125 SE 8101665 | BE 887991 ES 500397 JP 56167698 | CA 1198385 ES 510007 NL 8101289 |
| EP | 20147 | AU 58926/80 DK 2329/80 ES 492044 IL 60186 PL 224664 ZA 8002992 | CA 1194432 EG 15156 ES 499045 JP 56036499 PT 71323 | DD 202045 EP 20147 FI 801/43 NZ 1939867 YU 1453/80 |
| EP | 49945 | JP 57081415 | | |
| GB | 1529871 | AT 8293/75 DE 2547224 ES 442242 FR 2289206 NL 7512590 SE 7511907 | AU 86282/75 DK 4785/75 FI 752964 IL 58384 NO 753578 SE 8100404 | BE 835075 DK 2784/78 FI 782827 JP 51070810 NZ 179108 ZA 7506873 |
| AU | 34497/84 | EP 142192 | JP 60172932 | JP 60172933 |
| AU | 11990/83 | CA 1203164 JP 5822035 | DK 1130/83 ZA 8301463 | EP 88695 |